Antibacterial Agents

Merging Natural Products: Muraymycin–Sansanmycin Hybrid Structures as Novel Scaffolds for Potential Antibacterial Agents

Giuliana Niro, Stefanie C. Weck, and Christian Ducho*[a]

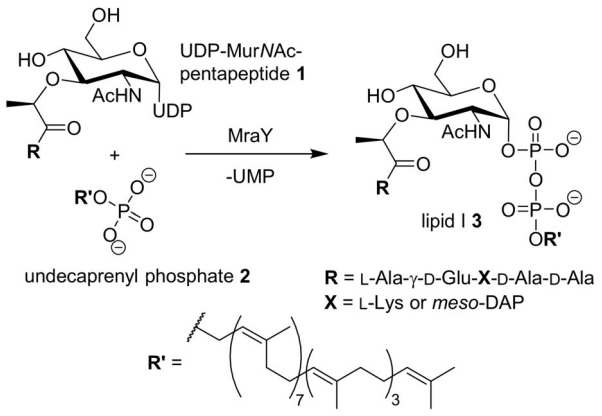
Abstract: To overcome bacterial resistances, the need for novel antimicrobial agents is urgent. The class of so-called nucleoside antibiotics furnishes promising candidates for the development of new antibiotics, as these compounds block a clinically unexploited bacterial target: the integral membrane protein MraY, a key enzyme in cell wall (peptidoglycan) biosynthesis. Nucleoside antibiotics exhibit remarkable structural diversity besides their uridine-derived core motifs. Some sub-classes also show specific selectivities towards different Gram-positive and Gram-negative bacteria, which are poorly understood so far. Herein, the synthesis of a novel hybrid structure is reported, derived from the 5′-defunctionalized uridine core moiety of muraymycins and the peptide chain of sansanmycin B, as a new scaffold for the development of antimicrobial agents. The reported muraymycin–sansanmycin hybrid scaffold showed nanomolar activity against the bacterial target enzyme MraY, but displayed no significant antibacterial activity against *S. aureus*, *E. coli*, and *P. aeruginosa*.

Introduction

With the rise of bacterial strains exhibiting resistance against established antibiotics, novel drug candidates are urgently needed for the future treatment of microbial infections.[1] Cell wall (i.e., peptidoglycan) biosynthesis is one typical bacterial pathway targeted by many established antibiotics.[2] However, nucleoside antibiotics are a promising class for the development of new therapeutics as they block a clinically unexploited target protein (MraY) involved in the membrane-associated stages of peptidoglycan biosynthesis. By the inhibition of MraY (translocase I), nucleoside antibiotics prevent the formation of lipid I **3** as a key intermediate of bacterial cell wall formation (Scheme 1). MraY is a membrane protein consisting of ten transmembrane helices and five cytosolic loops.[3] It catalyzes the transfer of UDP-Mur*N*Ac pentapeptide **1** ('Park's nucleotide') to the membrane-bound lipid carrier undecaprenyl phosphate **2**, thus yielding membrane-associated lipid I **3**.[4]

Uridine-derived nucleoside antibiotics all share MraY as their primary biological target, but are divided into structurally varying sub-classes, for example, muraymycins, mureidomycins, sansanmycins, caprazamycins, tunicamycins, and others.[5] Despite this similar mode of action, their antimicrobial activities differ significantly.[6] For instance, the pronounced activities of mureidomycins, sansanmycins, and their related classes against the Gram-negative ESKAPE pathogen *P. aeruginosa* distinguishes them from most other nucleoside antibiotics, including muraymycins, which are mainly active against Gram-positive pathogens.[7] Caprazamycins also display some limited anti-*Pseudomonas* activity, but primarily good activity against Gram-positive bacteria and *M. tuberculosis*. These pronounced differences in the antibacterial activities of different sub-classes of nucleoside antibiotics are only scarcely studied and therefore not well understood.

In the case of muraymycins, 19 naturally occurring congeners were originally isolated from a *Streptomyces* sp. in 2002.[5a]



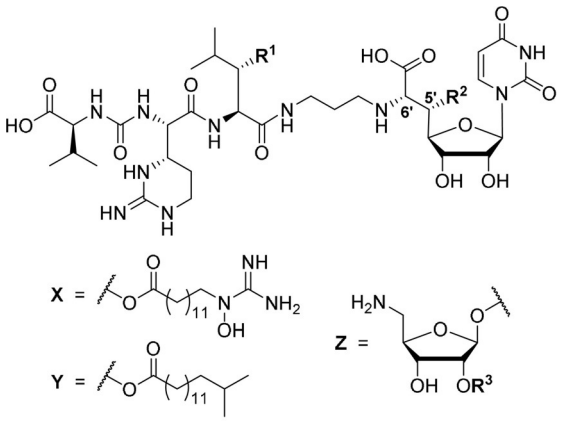
Scheme 1. MraY-catalyzed membrane-associated formation of lipid I **3** (membrane not shown). UDP = uridine diphosphate, UMP = uridine monophosphate, DAP = 2,6-diaminopimelic acid.

[a] *Dr. G. Niro, S. C. Weck, Prof. Dr. C. Ducho*
Department of Pharmacy, Pharmaceutical and Medicinal Chemistry
Saarland University
Campus C2 3, 66123 Saarbrücken (Germany)
E-mail: christian.ducho@uni-saarland.de

Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/chem.202003387.

Recently, additional muraymycins were isolated from the same producing strain.[5b] In general, muraymycins consist of a (5′*S*,6′*S*)-glycyluridine ('GlyU') moiety, which is attached to a urea peptide chain via an aminopropyl linker. The peptide moiety consists of an L-leucine derivative, the non-proteinogenic amino acid L-epicapreomycidine (as a cyclic arginine derivative), and a urea-linked L-valine unit (Figure 1). With respect to their detailed structural features, muraymycins are divided into four series (A–D). Series A–C contain a non-proteinogenic (3*S*)-3-hydroxy-L-leucine unit that is acylated with fatty acid moieties in the case of series A and B. Where congeners of series A contain ω-functionalized fatty acyl structures (e.g., A1 **4** and A5 **5**, Figure 1), derivatives of series B possess unfunctionalized, branched fatty acyl chains of different lengths (e.g., B8 **6**, Figure 1). The C series contains non-acylated (3*S*)-3-hydroxy-L-leucine, and the D series proteinogenic L-leucine. Unusual structural motifs, such as the 5′-*O*-aminoribosylated glycyluridine (GlyU) unit and the non-proteinogenic amino acids (3*S*)-3-hydroxy-L-leucine and L-epicapreomycidine, make the total synthesis of muraymycins challenging. However, the total synthesis of naturally occurring muraymycins of the D series has been reported by Ichikawa and Matsuda as well as by Kurosu and co-workers.[8] Further synthetic routes towards several building blocks of muraymycins have also been developed.[9]

We have established the synthesis of structurally simplified 5′-defunctionalized ('5′-deoxy') muraymycin analogs (e.g., the 5′-deoxy congener **9** of muraymycin C4 **7**, Figure 1).[10] Synthetic analog **9** was found to be a rather potent inhibitor of MraY in vitro ($IC_{50} = 95 \pm 19$ nM), although the aminoribose unit is known to mediate a key interaction with the target enzyme (see below).[11] The 5′-defunctionalized version of the GlyU core unit is synthetically more easily accessible,[12] thus making such 5′-deoxy analogs reasonable tools for structure–activity relationship (SAR) studies.[13] Further SAR investigations have been conducted on aminoribosylated muraymycin analogs[14a,b] as well as on structurally more distinct muraymycin-inspired derivatives.[14c]



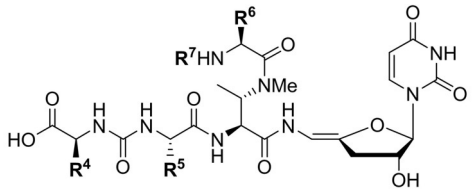
	R¹	R²	R³
muraymycin A1 **4**	X	Z	Me
muraymycin A5 **5**	X	OH	---
muraymycin B8 **6**	Y	Z	Me
muraymycin C4 **7**	OH	OH	---
muraymycin D2 **8**	H	Z	H
5′-deoxy C4 **9**	OH	H	---

Figure 1. Selected examples of naturally occurring muraymycins (**4**–**8**) as well as the synthetic 5′-deoxy analog **9** of muraymycin C4 **7**.

Mureidomycins, sansanmycins, and related classes contain a 3′-deoxyuridine-derived core moiety, which is attached (via a remarkable enamide linkage) to a non-proteinogenic (2*S*,3*S*)-2-amino-3-methylaminobutyric acid (AMBA) unit. The AMBA structure then effectively branches the peptide chain (Figure 2).[5c,d,15] In position **R⁴**, the aromatic side chains of amino acids such as tryptophan (e.g., **12** and **13**) or non-proteinogenic *m*-tyrosine (e.g., **10**, **11**, and **14**) are incorporated. In position **R⁵**, the side chains of hydrophobic amino acids (e.g., Met, Leu, or Ala) can be found. In position **R⁶**, a *m*-tyrosine side chain is present for mureidomycins and sansanmycins, but a methyl group (i.e., alanine) is present for pacidamycins (e.g., **14**). In the case of mureidomycin C **11**, an additional glycine unit is attached in position **R⁷**.

SAR studies on mureidomycins and related classes have shown that aromatic amino acids in position **R⁴** (Figure 2) are required for biological activity, whereas hydrophobic residues in position **R⁵** are preferred.[16a,b] In position **R⁶**, *m*-tyrosine is favored, however, sterically less bulky amino acids are tolerated as well.[16c] It has also been shown that the (2*S*,3*S*)-configuration of the AMBA moiety as well as the native L-configuration of all other amino acid units are crucial for antimicrobial activity of these MraY inhibitors.[16c,d] These observed trends are mainly based on MIC values against *P. aeruginosa* or *M. tuberculosis* though. It is therefore unclear if the lack of antimicrobial activity for other analogs might be a result of impaired MraY inhibition, hampered cellular uptake, or increased cellular efflux (as the active site of MraY is located at the cytosolic face of the membrane). A very recent SAR study on mureidomycin analogs also included some first insights into their selective cellular uptake into *P. aeruginosa*.[16e]

Following the first X-ray crystal structure of ligand-free MraY (from the extremophile *Aquifex aeolicus*),[17] Lee and co-workers reported the first X-ray co-crystal structure of MraY (again from *Aquifex aeolicus*) in complex with the inhibitor muraymycin D2



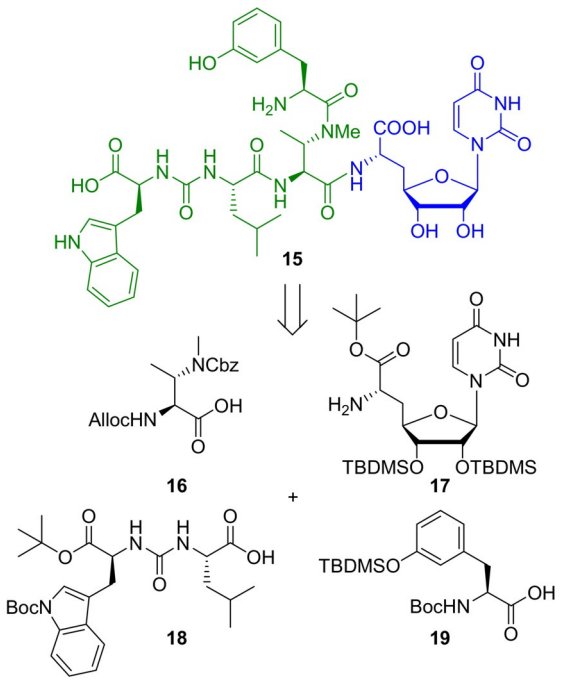
	R⁴	R⁵	R⁶	R⁷
mureidomycin A **10**	(*m*-Tyr)	(Met)	(*m*-Tyr)	H
mureidomycin C **11**	(*m*-Tyr)	(Met)	(*m*-Tyr)	Gly
sansanmycin A **12**	(Trp)	(Met)	(*m*-Tyr)	H
sansanmycin B **13**	(Trp)	(Leu)	(*m*-Tyr)	H
pacidamycin D **14**	(*m*-Tyr)	(Ala)	(Ala)	H

Figure 2. Selected examples of naturally occurring mureidomycins (**10**, **11**), sansanmycins (**12**, **13**), and pacidamycins (**14**). Three letter codes of amino acids in parentheses refer to the side chain of the respective amino acid.

8.[11a,b] A comparison with the X-ray crystal structure of the ligand-free apoprotein[17] revealed significant conformational changes upon inhibitor binding. Two defined binding pockets for the uracil and the 5′-aminoribose moieties were identified, whereas the peptide unit is positioned on the surface of the protein. Recently, Lee and colleagues reported an even more detailed and comprehensive binding model for the inhibition of MraY by several nucleoside antibiotics, based on a range of X-ray co-crystal structures.[11c] Thereby, they identified common binding sites addressed by multiple sub-classes of nucleoside antibiotics as well as unique sites for the binding of some sub-classes, all on the cytosolic side of MraY. Interestingly, the *m*-tyrosine moiety of mureidomycins was found to bind to the same pocket as the 5′-aminoribose moiety in the case of muraymycins (and related caprazamycins).[11c]

This recent comprehensive study on MraY inhibition by different types of nucleoside antibiotics strongly suggests that it might be possible to generate novel scaffolds for MraY inhibitors in a modular fashion. Thus, it might be assumed that, for instance, the nucleoside unit of one sub-class can be combined with the peptide unit of a different sub-class to generate a "hybrid" MraY inhibitor. This strategy might not only furnish new lead structures for antibacterial drug development, but it might also be useful to elucidate the selectivity in the antibacterial action of different sub-classes of nucleoside antibiotics. Based on these considerations, we have designed such an unprecedented hybrid structure, **15** (Scheme 2).

Target compound **15** contains the 5′-defunctionalized ('5′-deoxy') version of the GlyU core unit of muraymycins (like muraymycin analog **9**, highlighted in Scheme 2 in blue) and the peptide moiety of sansanmycin B **13** (highlighted in Scheme 2 in green, also see Figures 1 and 2). It should be noted that muraymycins usually show activity against Gram-positive bacteria, but not against the Gram-negative ESKAPE pathogen *P. aeruginosa*. On the other hand, sansanmycin B **13** exhibits anti-*Pseudomonas* activity (MIC: 4–16 μg mL^{-1}).[18] Overall, hybrid structure **15** is therefore potentially useful for the following purposes: (i) to test the aforementioned hypothesis if structural features of different sub-classes of nucleoside antibiotics can be combined in a modular fashion to furnish novel MraY inhibitors (i.e., as a 'proof-of-principle'); (ii) to test if the presence of structural features of one particular sub-class with activity against a particular pathogen might also make the overall hybrid structure active against that pathogen. In the case of **15**, this would mean that the presence of the sansanmycin peptide unit might potentially confer anti-*Pseudomonas* activity of **15**, in spite of the non-activity of muraymycins against *Pseudomonas*. In this work, we report the synthesis of novel hybrid structure **15** as well as its biological evaluation.



Scheme 2. Hybrid target structure **15**, designed based on 5′-defunctionalized muraymycin analogs (blue moiety) and sansanmycin B (green moiety), as well as retrosynthetic considerations for its preparation.

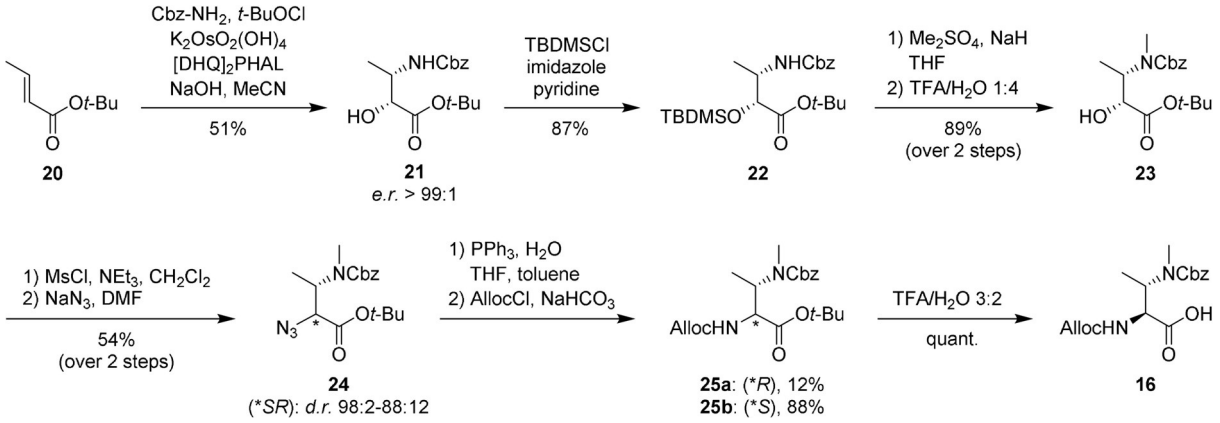
Results and Discussion

Synthesis

For the synthesis of hybrid structure **15**, we aimed for a modular approach that would also enable potential further structural variations in future studies. Therefore, it was envisioned to construct **15** from four individual building blocks **16**–**19** (Scheme 2). After the connection of all four building blocks towards the complete scaffold, a single global deprotection step under acidic conditions should then furnish target compound **15**.[8a,10,14]

For the synthesis of the protected nucleosyl amino acid building block **17**, we employed our previously reported protocol consisting of six steps starting from uridine (reactions not shown).[12b] The orthogonally protected version **16** of non-proteinogenic (2*S*,3*S*)-2-amino-3-methylaminobutyric acid (AMBA) was required to prepare the sansanmycin-derived "branched" peptide structure of the target compound. Building block **16** was therefore synthesized via a modified version of the route originally reported by Hennings and Williams (Scheme 3).[19]

First, stereoselective Sharpless aminohydroxylation of *tert*-butyl crotonate **20** (based on an established procedure[20]) was used to generate *syn*-configured amino alcohol **21** in 51% yield (Scheme 3). In principle, the determination of the absolute stereochemistry of **21** by using Mosher ester methodology has been reported before as well.[20] However, we now report full analytical data of the corresponding products. Therefore, both enantiomers of amino alcohol **21** were synthesized, analyzed by chiral HPLC, and transformed to the corresponding Mosher esters. The stereochemistry was then assigned based on ^{1}H NMR data (see the Supporting Information for a full description of this procedure). Subsequently, the hydroxy group of **21** was silyl-protected to give silyl ether **22** in 87% yield. Methylation of the carbamate nitrogen was conducted as reported,[19] even though a mixture of methylated silyl ether and the desilylated compound **23** was obtained. Therefore, the subsequent acidic desilylation step was carried out using this

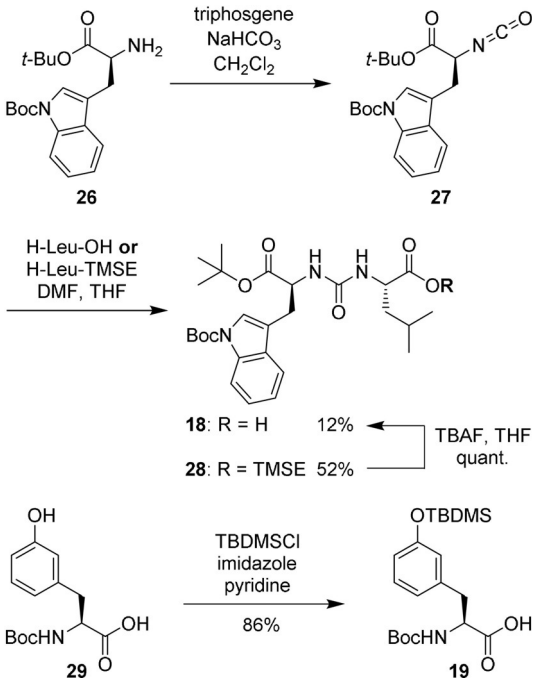


Scheme 3. Synthesis of the orthogonally protected (2*S*,3*S*)-2-amino-3-methylaminobutyric acid (AMBA) building block **16**.

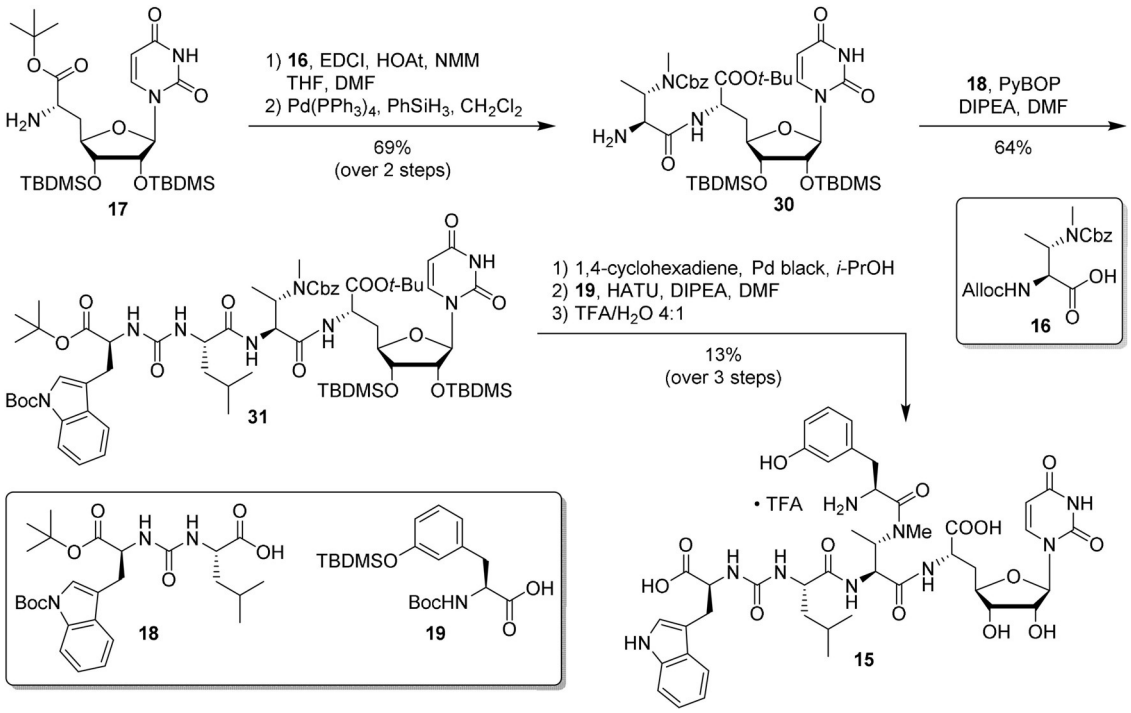
mixture to furnish the desired alcohol **23** in 89% yield over two steps from **22**. Azide **24** was then synthesized by nucleophilic substitution at the 2-position (after transformation of **23** into its mesylate) based on an established protocol.[19] However, the product (54% overall yield over two steps from **23**) was unexpectedly obtained as an epimeric mixture with varying diastereomeric ratios (d.r. 98:2 to 88:12), eventually owing to a partially occurring S_Ni mechanism. The epimeric mixture of azide **24** was not separated, but reduced under Staudinger conditions, and the resultant amine was directly allyloxycarbonyl-(Alloc)-protected. At this stage, chromatographic separation of the epimers was feasible and the desired (2*S*)-epimer, that is, *anti*-isomer **25b**, was obtained in 88% yield. Acidic cleavage of the *tert*-butyl ester then afforded the desired stereoisomerically pure building block **16** in quantitative yield (Scheme 3).

For the synthesis of the urea dipeptide unit, commercially available Boc-protected tryptophan *tert*-butyl ester **26** was transformed into the corresponding isocyanate **27** by using triphosgene under basic conditions (Scheme 4). The highly reactive isocyanate **27** was not purified, but directly converted into the corresponding urea dipeptide by the addition of a second amino acid. This procedure avoided epimerization reactions and thus furnished the resultant urea dipeptide products in stereoisomerically pure form. In a first attempt, unprotected L-leucine was used as the second amino acid, but only poor yields (i.e., 12%) of the desired building block **18** were achieved, most likely owing to solubility issues. To overcome this hurdle, the 2-(trimethylsilyl)ethyl (TMSE) ester of L-leucine was used as the second amino acid instead. This variant afforded urea dipeptide TMSE ester **28** in an improved yield of 52%. The L-leucine TMSE ester had been synthesized as reported before.[21] Subsequent cleavage of the TMSE ester by using tetra-*n*-butylammonium fluoride (TBAF) for desilylation furnished the desired building block **18** in quantitative yield (Scheme 4). Hence, the two-step route via the TMSE ester intermediate **28** was found to be superior to the one-step transformation into **18** by using unprotected L-leucine. The last required building block **19** was synthesized by TBDMS-protection of commercially available *N*-Boc-protected *m*-tyrosine **29** under standard conditions, affording product **19** in 86% yield (Scheme 4).

With all building blocks **16**–**19** in hand, the full-length scaffold of target structure **15** was constructed in a stepwise manner (Scheme 5). The AMBA-derived building block **16** was attached to the 5′-deoxy nucleosyl amino acid **17** by standard amide coupling using EDCI and HOAt for activation.[16b] The desired coupling product was obtained in 94% yield and subsequently submitted to Alloc cleavage under usual Pd-catalyzed conditions.[22] The resultant amine **30** was isolated in 73% yield (69% over two steps from **17**). The urea dipeptide moiety was attached next, by using building block **18** and PyBOP as a coupling reagent. The reaction was carefully monitored by LC-MS analysis. The desired product **31** was then obtained in 64%



Scheme 4. Synthesis of the urea dipeptide building block **18** and of the protected *m*-tyrosine building block **19**.



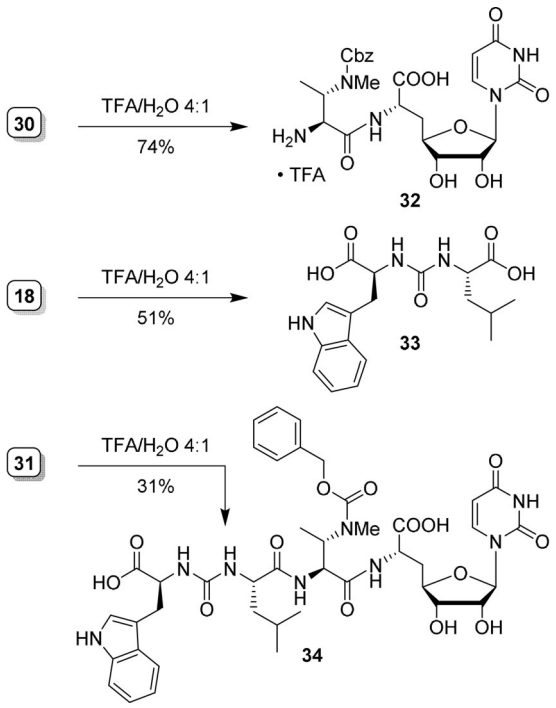
Scheme 5. Endgame of the synthesis of target structure **15**: connection of building blocks **16**–**19** and acidic global deprotection.

yield after aqueous workup and chromatographic purification. Finally, the secondary amine was Cbz-deprotected under transfer hydrogenation conditions (1,4-cyclohexadiene and Pd black in *iso*-propanol) to avoid unwanted reduction of the uracil C5-C6 double bond.[12b,13b] The identity of the obtained product was confirmed by LC-MS analysis only, and the secondary amine was directly submitted to a final amide coupling with the *m*-tyrosine building block **19**. This reaction was hampered owing to steric hindrance of the secondary amine, and its conversion was closely monitored by LC-MS analysis. However, an excess of the carboxylic acid **19**, HATU and DIPEA was used, and with elongated reaction time and slightly elevated temperature, the coupling product was finally obtained. This fully protected derivative of the hybrid structure **15** was not characterized, but directly submitted to acidic global deprotection by using aqueous trifluoroacetic acid. Target compound **15** was isolated (after purification by semi-preparative HPLC) in 13% yield over the three final steps from intermediate **31** (Scheme 5).

Synthetic key intermediates **18**, **30**, and **31** were also fully deprotected under the same acidic conditions, to obtain additional target compounds **32**–**34** (in yields of 31–74%) and to evaluate their inhibitory potency towards the bacterial target enzyme MraY (Scheme 6).

Biological evaluation

Target hybrid structure **15** as well as additional target compounds **32**–**34** were investigated for their in vitro inhibitory potency against the bacterial enzyme MraY, by using an established fluorescence-based assay.[23] As reported before, a crude membrane preparation of MraY from *S. aureus* (heterologously overexpressed in *E. coli*) was used as source of MraY activity.[5b,10,21,23] The obtained inhibitory activities (IC_{50} values) are listed in Table 1. It cannot totally be ruled out that these inhibitory potencies towards MraY might be different with MraY ho-



Scheme 6. Acidic deprotection of key intermediates **18**, **30**, and **31** towards additional target compounds **32**–**34**.

Table 1. In vitro inhibitory activities of **15** and **32–34** against MraY.

Compound	IC_{50} [μM][a]
15	0.71 ± 0.17
32	~1100 ± 300
33	~1200 ± 500
34	4.4 ± 0.6
9	0.095 ± 0.019[b]

[a] MraY from *S. aureus*, heterologously overexpressed in *E. coli*, crude membrane preparation. [b] See ref. [23e].

mologs from other bacterial species. However, previous results strongly suggest that the similarity of different MraY congeners is quite pronounced and that differences in IC_{50} values are much more dependent on the choice of MraY preparation (crude membranes vs. solubilized protein) rather than the employed MraY homolog.[23e]

The full-length muraymycin–sansanmycin hybrid structure **15** showed MraY inhibition in the high-nanomolar range (IC_{50} = 0.71 ± 0.17 μM). This proved our general concept that structural motifs of different sub-classes of nucleoside antibiotics can be combined in a modular fashion to generate a novel scaffold with inhibitory activity towards MraY as the bacterial target protein. In comparison, our previously reported synthetic muraymycin analog **9**[10,23e] (see above) had been found to be an approximately 7-fold more active MraY inhibitor (Table 1). However, if **15** is interpreted as a "hit" compound with respect to its novel modular scaffold, this result is actually rather encouraging, having in mind that **9** closely resembles a naturally occurring nucleoside antibiotic (i.e., muraymycin C4 **7**). Thus, a future structural optimization of **15** might lead to even more potent MraY inhibitors. Unfortunately, only MIC values for the inhibition of bacterial growth, but usually no IC_{50} values for MraY inhibition have been described for most previously reported sansanmycins and their analogs.[16b,24] This makes a comparison of the inhibitory potency of **15** with the activity of sansanmycins difficult.

Owing to its full-length scaffold, additional analog **34** can be seen as a congener of **15**. It was found to inhibit MraY at low micromolar concentrations (IC_{50} = 4.4 ± 0.6 μM), that is, as an approximately 6-fold weaker inhibitor relative to **15**. The additional target affinity of **15** clearly results from the formal exchange of the Cbz protecting group in **34** to the *m*-tyrosine unit in **15**, hence, from the additional presence of the phenolic hydroxy group and/or the primary amine. It is quite surprising that these structural alterations only furnish an approximately 6-fold improvement in activity with respect to Lee's recently reported structural model for MraY inhibition.[11c] Based on this work, one would expect that the primary amine of the *m*-tyrosine moiety in **15** should interact with the so-called "uridine-adjacent pocket" of MraY, which is also addressed by the aminoribose unit of naturally occurring muraymycins. In the case of muraymycins, the presence of the aminoribose unit leads to a boost in MraY inhibition.[23e] This suggests that the primary amine in **15** might not be ideally spatially oriented yet to mediate a more pronounced interaction with the uridine-adjacent pocket. In the case of 3′-hydroxymureidomycin A (i.e., a 3′-hydroxylated analog of **10** with a *m*-tyrosine unit at the same position as in **15**), hydrogen bonds of the hydroxy group and the amine of the *m*-tyrosine moiety with T75 and D265 of MraY have been observed by X-ray crystallography.[11c] On the other hand, the fact that **34** also was found to be a reasonably potent MraY inhibitor further supports our hypothesis that structural variations on the newly established scaffold of type **15** will be feasible.

Truncated analogs **32** and **33** can be interpreted as fragments of the full-length structure **34**. Both **32** and **33** were found to be very weak MraY inhibitors with in vitro activities in the low-millimolar range (Table 1). This highlights the relevance of a connection between the nucleoside unit and the peptide moiety to the full-length scaffold. On the other hand, it was fairly remarkable that some residual inhibitory activity towards MraY could still be detected for both truncated analogs **32** and **33**. This suggests that fragment-based approaches for the development of novel MraY inhibitors might be feasible.

Both full-length muraymycin–sansanmycin hybrid structures **15** and **34** were also tested for their activity as inhibitors of antibacterial growth *in cellulo*. However, no antibacterial activity (MIC > 50 μg mL^{-1}) was found against Gram-positive *S. aureus*, Gram-negative *E. coli* (efflux-competent DH5α and efflux-deficient Δ*tolC* strains), and the Gram-negative ESKAPE pathogen *P. aeruginosa*. This might be a result of limited cellular uptake, in particular as no growth inhibition was observed for the efflux-deficient *E. coli* strain Δ*tolC*. This result suggests that the novel scaffold of type **15** will also require structural optimization towards improved cellular uptake, even though the proof-of-principle for the MraY inhibition of such hybrid structures has been successfully provided. Hence, it cannot be evaluated yet if the presence of the sansanmycin peptide unit within the hybrid structure might potentially confer anti-*Pseudomonas* activity. Optimization of **15** towards improved cellular uptake might include i) decoration with lipophilic moieties;[14,23e] ii) conjugation to a siderophore;[25a] iii) functionalization with basic groups.[25b]

Conclusion

We herein report the synthesis and biological evaluation of the first artificial hybrid structures of two sub-classes of nucleoside antibiotics. We have formally combined the 5′-defunctionalized version of the muraymycin nucleoside unit with the peptide moiety of sansanmycin B. The synthesis of hybrid structure **15** was accomplished in 1.3% yield over 14 linear steps, by using a modular approach involving four building blocks. This modular route is expected to be useful for future structural variations on the scaffold of type **15** for more detailed SAR studies. Hybrid structure **15** was found to be a reasonably potent (i.e., high nanomolar) inhibitor of the bacterial target protein MraY in vitro, thus furnishing the attempted proof-of-principle that structural features of different sub-classes of nucleoside antibiotics can be combined in a modular fashion to furnish novel MraY inhibitors. Additionally synthesized full-length analog **34** (as a structurally slightly simplified congener of **15**) also inhibit-

ed MraY in vitro. This result supports the hypothesis that further structural variations of the hybrid scaffold will be feasible and can be expected to afford more derivatives of this type with inhibitory activity towards MraY. More detailed studies on hybrid structures of type **15** will be required for further SAR insights and optimization of the target interaction, but also to obtain analogs with antibacterial activity *in cellulo* (which is absent yet for both **15** and **34**). Overall, we have therefore demonstrated that the modular generation of novel scaffolds for MraY inhibitors is feasible, even though it apparently furnishes "hit" compounds rather than elaborated MraY-inhibiting antibiotics with antibacterial activity *in cellulo*. The reported approach to fuse structural features of nucleoside antibiotics into hybrid scaffolds is therefore expected to potentially provide new chemical entities for the development of MraY-inhibiting antimicrobial drug candidates.

Experimental Section

General methods

All chemicals, starting materials, and reagents were purchased from standard suppliers and used without further purification. *N*-Boc-L-*m*-tyrosine **29** was purchased from GL Biochem Shanghai. Building block **17** was synthesized from uridine as reported before.[12b] Air- and/or water-sensitive reactions were carried out under nitrogen atmosphere with anhydrous solvents if not indicated otherwise. Anhydrous solvents were obtained in the following manner: THF, DMF, and CH_2Cl_2 were dried with a solvent purification system (MBRAUN MB SPS 800). Pyridine and *i*PrOH were dried over CaH_2 and distilled. All of the thus obtained solvents were stored over molecular sieves (4 Å). All other solvents were of technical quality and distilled prior to use, and deionized water was used throughout. The glass equipment was dried by heating in vacuo prior to use. The nitrogen used was dried by using orange gel and phosphorus pentoxide. All reactions were monitored by TLC if not stated otherwise. TLC was performed on aluminium plates precoated with silica gel 60 F254 (VWR). Visualization of the spots was carried out by using UV light (254 nm) and/or staining with VSS TLC stain (4 g vanillin, 25 mL H_2SO_4 (conc.), 80 mL AcOH, all dissolved in MeOH (680 mL)) under heating. R_f values are given to the nearest 0.05. For column chromatography, silica gel 60 (40–63 μm, 230–400 mesh ASTM, VWR) was used. Preparative centrifugal TLC was carried out with a Chromatotron™ 7924T by T-Squared Technology, using glass plates coated with silica gel 60 PF_{254} containing a fluorescent indicator (VWR, thickness depending on the amount of crude material to be separated, for 50–500 mg: 1 mm layer). Semipreparative HPLC was performed with a VWR-Hitachi system equipped with an L-2300 pump, an L-2200 autosampler, an L-2455 Diode Array Detector (DAD), and a LichroCart™ Purospher™ RP18e column (5 μm, 10×250 mm, VWR). Method 1: eluent A water (+0.1% TFA), eluent B MeCN (+0.1% TFA); 0–3 min gradient of B (30–40%), 3–22 min gradient of B (40–60%), 22–34 min 60% B, 34–37 min gradient of B (60–100%), 37–39 min 100% B, 39–40 min gradient of B (100–30%), flow 2.5 mL min^{-1}, UV/Vis detector (254 nm, 260 nm). Method 2: eluent A water (+0.1% TFA), eluent B MeCN (+0.1% TFA); 0–30 min gradient of B (1–20%), 30–34 min gradient of B (20–100%), 34–44 min 100% B, 44–45 min gradient of B (100–1%), flow 2.5 mL min^{-1}, UV/Vis detector (254 nm, 260 nm). Method 3: eluent A water (+0.1% TFA), eluent B MeCN (+0.1% TFA); 0–8 min gradient of B (20–36%), 8–32 min 36% B, 32–38 min gradient of B (36–100%), 38–44 min 100% B, 44–45 min gradient of B (100–20%), flow 2.5 mL min^{-1}, UV/Vis detector (254 nm, 260 nm). Method 4: eluent A water (+0.1% TFA), eluent B MeCN (+0.1% TFA); 0–8 min gradient of B (20–28%), 8–32 min 28% B, 32–38 min gradient of B (28–100%), 38–44 min 100% B, 44–45 min gradient of B (100–20%), flow 2.5 mL min^{-1}, UV/Vis detector (254 nm, 260 nm). NMR spectra were recorded by using the following Bruker NMR spectrometers: for ^{1}H NMR spectra at 500 MHz and ^{13}C NMR spectra at 126 MHz: Avance I 500 with a B ACS 60 auto sampler, Avance DRX 500 or Avance III 500 with a TCI cryo probe head; for ^{19}F NMR spectra at 376 MHz: Avance II 400. For the assignment of signals, ^{1}H,^{1}H-COSY, ^{1}H,^{13}C-HSQC, and ^{1}H,^{13}C-HMBC spectra were used. In the case of low signal-to-noise ratios, some chemical shifts were derived from those 2D spectra. All ^{13}C and ^{19}F NMR spectra are ^{1}H-decoupled. All spectra were recorded at room temperature if not indicated otherwise and were referenced internally to solvent residual signals wherever possible. Chemical shifts (δ) are quoted in ppm and coupling constants (J) are reported in Hz. $^+$ Indicates additional NMR signals from *N*-methylated compounds that formed rotamers (marked wherever possible). § Indicates that NMR data are given for the major rotamer. In these cases, NMR measurements at high temperatures were not feasible owing to decomposition of the compound at elevated temperatures. Low-resolution mass spectra were recorded with a liquid chromatography coupled mass spectrometer (LC-MS) Surveyor MSQ Plus from Finnigan. For the LC separation prior to detection, a Nucleodur™ 100-5 C_{18} column (5 μm, 3×125 mm) was used. High-resolution mass spectra were recorded with a Thermo Scientific Q Exactive Orbitrap mass spectrometer with ESI ionization mode coupled with an Ultimate 3000 HPLC system by Thermo Scientific, equipped with a Thermo Accucore™ phenyl-X column (2.1 μm, 3×100 mm). UV spectroscopy was carried out with an Agilent Cary 100 spectrophotometer. Absorption maxima of HPLC-purified compounds were determined from DAD data. Infrared spectroscopy of pure compounds was performed with a Bruker Fourier transform infrared (FTIR) spectrometer ALPHA with an integrated PlatiniumATR™ unit. Specific optical rotations were determined by using a P3000 polarimeter by Krüss (λ = 589 nm) in the indicated solvents at room temperature.

Target hybrid structure (15)

To a solution of **31** (20 mg, 15 μmol) in *i*PrOH (2.0 mL), Pd black (spatula tip) and 1,4-cyclohexadiene (0.14 mL, 0.15 mmol) were added. The resultant suspension was stirred under nitrogen atmosphere at room temperature for 5 h. After full conversion had been observed by TLC, the mixture was filtered through a syringe filter, which was then washed with EtOAc. The solvent of the combined filtrates was evaporated under reduced pressure to give the crude amine, which was used without further purification. Protected L-*m*-tyrosine **19** (11 mg, 27 μmol) was dissolved in DMF (2 mL). At 0 °C, *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU; 12 mg, 32 μmol) was added, the mixture was allowed to warm to room temperature and stirred for a further 15 min. Then, a solution of the crude amine in DMF (3 mL) and *N*,*N*-diisopropylethylamine (DIPEA; 12 μL, 67 μmol) were added. The reaction mixture was stirred at room temperature for 22 h. As LC-MS analysis still indicated major amounts of unreacted amine, additional **19** (11 mg, 27 μmol), DIPEA (12 μL, 67 μmol) and HATU (9 mg, 24 μmol) were added and the solution was stirred at room temperature for further 9 h. As the conversion was still incomplete based on LC-MS analysis, the mixture was heated to 35 °C and stirred at this temperature for an additional 15 h until full conversion of the amine was observed by LC-MS. Then, EtOAc (30 mL)

was added and the solution was washed with HCl (1 M, 30 mL), water (5×30 mL), and brine (30 mL), dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The resultant crude product was purified by flash column chromatography (CH_2Cl_2/MeOH 98:2). The thus obtained crude fully protected intermediate was analyzed by LC-MS only. It was then dissolved in TFA and water (4:1, 2.5 mL) and stirred at room temperature for 26 h. Water (5 mL) was added and the solvent was evaporated by lyophilization. The resultant crude product was purified by semi-preparative HPLC (method 4) to give **15** as a slightly yellowish fluffy solid (2.1 mg, 13% over 3 steps from **31**). ^{1}H NMR (500 MHz, CD_3OD): δ = 0.77 (d, J = 6.6 Hz, 3H, AMBA-H-4), 0.89–0.94 (m, 6H, Leu-H-5), 1.41–1.53 (m, 2H, Leu-H-3), 1.61–1.71 (m, 1H, Leu-H-4), 2.05–2.14$^+$, 2.15–2.22 (m, 1H, H-5′a), 2.25–2.33 (m, 1H, H-5′b), 2.79 (s, 3H, NCH_3), 2.81–2.85 (m, 1H, *m*-Tyr-H-3a), 3.11–3.29 (m, 3H, Trp-H-3, *m*-Tyr-H-3b), 3.86–3.95 (m, 1H, H-3′), 3.95–4.02$^+$, 4.02–4.07 (m, 1H, H-4′), 4.14–4.17 (m, 3H, H-2′, AMBA-H-3, Leu-H-2), 4.40 (dd, J = 9.1, 5.3 Hz, 1H, *m*-Tyr-H-2), 4.53 (dd, J = 6.9, 4.8 Hz, 1H, AMBA-H-2), 4.55–4.59 (m, 2H, H-6′, Trp-H-2), 4.60–4.68$^+$ (m, 1H, H-6′), 5.67$^+$, 5.70 (d, J = 8.1 Hz, 1H, H-5), 5.71$^+$, 5.75 (d, J = 4.1 Hz, 1H, H-1′), 6.66–6.68 (m, 1H, *m*-Tyr-aryl-H-2), 6.72–6.78 (m, 2H, *m*-Tyr-aryl-H-4, *m*-Tyr-aryl-H-6), 6.99 (ddd, J = 8.0, 7.4, 1.0 Hz, 1H, Trp-indole-H-6), 7.06 (dd, J = 8.1, 1.0 Hz, 1H, Trp-indole-H-5), 7.08$^+$, 7.10 (s, 1H, Trp-indole-H-2), 7.18 (dt, J = 7.8, 1.8 Hz, 1H, *m*-Tyr-aryl-H-5), 7.29–7.34 (m, 1H, Trp-indole-H-4), 7.52–7.57 (m, 2H, H-6, Trp-indole-H-7), 7.60$^+$ (d, J = 8.1 Hz, 1H, H-6) ppm; ^{13}C NMR (126 MHz, CD_3OD): δ = 13.45, 14.72$^+$ (AMBA-C-4), 22.08$^+$, 23.48 (Leu-C-5), 23.48$^+$, 23.56 (Leu-C-5), 25.84 (Leu-C-4), 28.50 (NCH_3), 28.83$^+$, 29.01 (Trp-C-3), 35.67$^+$, 35.77 (C-5′), 37.38 (*m*-Tyr-C-3), 42.05$^+$, 42.10 (Leu-C-3), 51.06$^+$, 51.33 (C-6′), 53.79$^+$, 53.83 (Leu-C-2), 54.03 (*m*-Tyr-C-2), 54.63$^+$, 54.80, 55.04, 56.13$^+$, 56.42 (AMBA-C-2, AMBA-C-3, Trp-C-2), 74.70 (C-2′), 74.73 (C-3′), 81.60$^+$, 81.65 (C-4′), 92.20$^+$, 92.47 (C-1′), 102.87$^+$, 102.95 (C-5), 110.52$^+$, 110.61 (Trp-indole-C-3′), 112.20 (Trp-indole-C-4), 115.85$^+$, 115.90 (*m*-Tyr-aryl-C-4), 117.42$^+$, 117.50 (*m*-Tyr-aryl-C-2), 119.49$^+$, 119.56 (Trp-indole-C-7), 119.84 (Trp-indole-C-6), 121.44$^+$, 121.56 (*m*-Tyr-aryl-C-6), 122.34$^+$, 122.39 (Trp-indole-C-5), 124.70$^+$, 124.72 (Trp-indole-C-2), 128.91$^+$, 128.98 (Trp-indole-C-3a), 131.32$^+$, 131.39 (*m*-Tyr-aryl-C-5), 138.00$^+$, 138.04 (Trp-indole-C-7a), 142.90$^+$, 142.97 (C-6), 152.18$^+$, 152.25 (C-2), 159.22$^+$, 159.30 (urea-C=O), 160.00 (*m*-Tyr-aryl-C-1′), 162.28$^+$, 162.56 (*m*-Tyr-aryl-C-3), 166.17$^+$, 166.20 (C-4), 169.99 (AMBA-C-1), 170.24 (C-7′), 171.48 (Leu-C-1), 174.59 (Trp-C-1), 176.28 (C-7′) ppm; ^{19}F NMR (376 MHz, CD_3OD): δ = −77.14 (TFA-CF_3) ppm; MS (ESI): m/z = 922.71 $[M+H]^+$; HRMS (ESI): calcd for $C_{43}H_{56}N_9O_{14}$ $[M+H]^+$ 922.3941; found: 922.3930; UV (HPLC, MeCN/H_2O): λ_{max} = 219, 266 nm; HPLC (method 4): t_R = 18.8 min.

(2*S*,3*S*)-2-(((Allyloxy)carbonyl)amino)-3-(((benzyloxy)carbonyl)(methyl)amino)butanoic acid (16)

A solution of **25b** (152 mg, 0.376 mmol) in trifluoroacetic acid and water (3:2, 10 mL) was stirred at room temperature for 3 d. The solvent was then evaporated under reduced pressure. The resultant crude product was purified by flash column chromatography (CH_2Cl_2/MeOH, 98:2 + 1% HCOOH) to give **16** as a highly viscous colorless oil (131 mg, quant.). $[\alpha]_D^{20}$ = −11.6 (c = 0.86, CH_2Cl_2); ^{1}H NMR (500 MHz, $C_2D_2Cl_4$, 80 °C): δ = 1.24 (d, J = 6.9 Hz, 3H, H-4), 2.83 (s, 3H, NCH_3), 4.40–4.48 (m, 2H, H-2, H-3), 4.51–4.54 (m, 2H, Alloc-H-1), 5.09 (d, J = 12.6 Hz, 1H, Cbz-H-1a), 5.12 (d, J = 12.6 Hz, 1H, Cbz-H-1b), 5.15 (dd, J = 10.5, 1.4 Hz, 1H, Alloc-H-3a), 5.26 (dd, J = 17.2, 1.4 Hz, 1H, Alloc-H-3b), 5.46 (brs, 1H, NH), 5.82–5.91 (m, 1H, Alloc-H-2), 7.21–7.32 (m, 5H, aryl-H) ppm; ^{13}C NMR (126 MHz, $C_2D_2Cl_4$, 80 °C): δ = 14.57 (C-4), 30.03 (NCH_3), 53.91 (C-3), 57.03 (C-2), 66.41 (Alloc-C-1), 67.85 (Cbz-C-1), 117.84 (Alloc-C-3), 127.95 (Cbz-C-3, Cbz-C-7), 128.26 (Cbz-C-5), 128.74 (Cbz-C-4, Cbz-C-6), 132.33 (Alloc-C-2), 136.79 (Cbz-C-2), 155.62 (Alloc-C=O), 156.05 (Cbz-C=O), 172.01 (C-1) ppm; MS (ESI): m/z = 373.14 $[M+Na]^+$; HRMS (ESI): calcd for $C_{17}H_{23}N_2O_6$ $[M+H]^+$ 351.1551; found: 351.1549; IR (ATR): $\tilde{\nu}$ = 2942, 1691, 1530, 1405, 1322, 1167, 932, 697 cm^{-1}; UV (MeCN): λ_{max} = 229, 257 nm; TLC (CH_2Cl_2/MeOH, 98:2 + 1% HCOOH): R_f = 0.30.

Protected L-trypotophan-L-leucine urea dipeptide (18)

Route 1: Boc-protected tryptophan *tert*-butyl ester hydrochloride **26** (300 mg, 0.756 mmol) was dissolved in CH_2Cl_2 and aq. $NaHCO_3$ solution (1:1, 10 mL), cooled to 0 °C and stirred vigorously. Triphosgene (76.2 mg, 0.269 mmol) was added in one portion and the mixture was stirred for 30 min. The aqueous layer was then extracted with CH_2Cl_2 (3×40 mL). The combined organics were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The resultant crude isocyanate **27** was directly used in the next reaction without further purification. To a suspension of L-leucine (99 mg, 0.76 mmol) in DMF (3 mL), a solution of the crude isocyanate **27** in THF (6 mL) was added at room temperature. The reaction mixture was stirred at room temperature for 21 h. Subsequently, EtOAc (50 mL) was added and the mixture was washed with HCl (1 M, 3×30 mL), dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The resultant crude product was purified by preparative centrifugal TLC (CH_2Cl_2/MeOH gradient) to give **18** as a white solid (48 mg, 12% over 2 steps from **26**).

Route 2: A solution of **28** (34 mg, 55 μmol) in THF (3 mL) was cooled to 0 °C and stirred at this temperature for 10 min. Then, TBAF solution (1 M in THF, 66 μL, 66 μmol) was added, the reaction mixture was allowed to warm to room temperature and stirred for 4 h. The solvent was evaporated under reduced pressure. The resultant crude product was purified by flash column chromatography (CH_2Cl_2/MeOH, 98:2→9:1) to give **18** as a white solid (29 mg, quant.). $[\alpha]_D^{20}$ = +11.3 (c = 0.47, CH_2Cl_2); ^{1}H NMR (500 MHz, [D_6]DMSO): δ = 0.83 (d, J = 3.3 Hz, 3H, Leu-H-5a), 0.85 (d, J = 3.3 Hz, 3H, Leu-H-5b), 1.27 (s, 9H, $OC(CH_3)_3$), 1.28–1.35 (m, 1H, Leu-H-3a), 1.45–1.52 (m, 1H, Leu-H-3b), 1.60 (s, 9H, Boc-$OC(CH_3)_3$), 1.63–1.70 (m, 1H, Leu-H-4), 2.96 (d, J = 7.0 Hz, 2H, Trp-H-3), 4.30 (dd, J = 13.1, 8.1 Hz, 1H, Leu-H-2), 4.34 (dd, J = 7.1, 7.0 Hz, 1H, Trp-H-2), 6.29 (brs, 1H, Trp-2-NH), 6.71 (brs, 1H, Leu-2-NH), 7.23–7.26 (m, 1H, Trp-indole-H-5), 7.28–7.38 (m, 1H, Trp-indole-H-6), 7.46 (s, 1H, Trp-indole-H-2), 7.60 (d, J = 7.5 Hz, 1H, Trp-indole-H-4), 8.02 (d, J = 8.2 Hz, 1H, Trp-indole-H-7) ppm; ^{13}C NMR (126 MHz, [D_6]DMSO): δ = 22.40 (Leu-C-5), 23.30 (Leu-C-5), 24.27 (Leu-C-4), 27.45, 27.69, ($OC(\underline{C}H_3)_3$, Boc-$OC(\underline{C}H_3)_3$, Leu-C-3), 43.29 (Trp-C-3), 52.92 (Leu-C-2), 53.64 (Trp-C-2), 80.14 ($O\underline{C}(CH_3)_3$), 83.53 (Boc-$O\underline{C}(CH_3)_3$), 114.62 (Trp-indole-C-7), 116.43 (Trp-indole-C-3), 119.35 (Trp-indole-C-5), 122.57 (Trp-indole-C-5), 123.81 (Trp-indole-C-2), 124.36 (Trp-indole-C-6), 130.23 (Trp-indole-C-3a), 134.64 (Trp-indole-C-7a), 149.01 (Boc-C=O), 157.22 (urea-C=O), 171.83 (Leu-C-1, Trp-C-1) ppm; MS (ESI): m/z = 518.32 $[M+H]^+$; HRMS (ESI): calcd for $C_{27}H_{40}N_3O_7$ $[M+H]^+$ 518.2861; found: 518.2845; UV (MeCN): λ_{max} = 230, 260, 286, 294 nm; IR (ATR): $\tilde{\nu}$ = 3301, 2967, 1730, 1552, 1368, 1255, 1154, 1085, 749 cm^{-1}; TLC (CH_2Cl_2/MeOH, 98:2 + 0.5% HCOOH): R_f = 0.15.

N-Boc-*O*-(*tert*-butyldimethylsilyl)-L-*m*-tyrosine (19)

N-Boc-L-*m*-tyrosine **29** (200 mg, 0.711 mmol) was dissolved in pyridine (3 mL). At 0 °C, imidazole (155 mg, 2.27 mmol) and *tert*-butyldimethylsilyl chloride (TBDMS chloride; 226 mg, 1.50 mmol) were added. The reaction mixture was allowed to warm to room temperature and stirred for 48 h. Subsequently, water (40 mL) and EtOAc (40 mL) were added. The organic layer was washed with brine (2×40 mL), dried over Na_2SO_4, and the solvent was evaporated under reduced pressure.

The resultant crude product was purified by flash column chromatography (CH_2Cl_2/MeOH, 99:1 + 1% HCOOH) to give **19** as a yellowish foam (242 mg, 86%). $[\alpha]_D^{20} = -2.1$ ($c = 1.4$, CH_2Cl_2); ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 0.16$ (s, 6H, $SiCH_3$), 0.94 (s, 9H, $SiC(CH_3)_3$), 1.32 (s, 9H, $OC(CH_3)_3$), 2.80 (dd, $J = 13.4$, 8.2 Hz, 1H, H-3a), 3.01 (dd, $J = 13.4$, 4.1 Hz, 1H, H-3b), 3.87–4.02 (m, 1H, H-2), 6.41 (d, $J = 6.4$ Hz, 1H, NH), 6.63 (d, $J = 7.9$ Hz, 1H, *m*-Tyr-aryl-H-4), 6.68 (s, 1H, *m*-Tyr-aryl-H-2), 6.80 (d, $J = 7.7$ Hz, 1H, *m*-Tyr-aryl-H-6), 7.09 (dd, $J = 7.9$, 7.7 Hz, 1H, *m*-Tyr-aryl-H-5) ppm; ^{13}C NMR (76 MHz, $CDCl_3$): $\delta = -4.54$ ($SiCH_3$), −4.50 ($SiCH_3$), 17.91 ($SiC(CH_3)_3$), 25.59 ($SiC(CH_3)_3$), 28.14 ($OC(CH_3)_3$), 36.17 (C-3), 55.02 (C-2), 77.94 ($OC(CH_3)_3$), 117.73 (*m*-Tyr-aryl-C-4), 120.73 (*m*-Tyr-aryl-C-2), 122.27 (*m*-Tyr-aryl-C-6), 129.17 (*m*-Tyr-aryl-C-5), 139.75 (*m*-Tyr-aryl-C-1), 154.85 (Boc-C=O), 155.35 (*m*-Tyr-aryl-C-3), 173.56 (C-1) ppm; MS (ESI): $m/z = 396.21$ $[M+H]^+$; HRMS (ESI): calcd for $C_{20}H_{34}NO_5Si$ $[M+H]^+$ 396.2201; found: 396.2228; IR (ATR): $\tilde{\nu} = 2934$, 2858, 1670, 1591, 1441, 1260, 1162, 840, 778 cm^{-1}; UV (MeCN): $\lambda_{max} =$ 223, 270 nm; TLC (CH_2Cl_2/MeOH, 97:3 + 1% HCOOH): $R_f = 0.30$.

tert-Butyl (2*R*,3*S*)-3-(((benzyloxy)carbonyl)amino)-2-hydroxybutanoate (21)

Aq. NaOCl solution (5%, 50 mL, 42 mmol) was cooled to 0 °C. Under the exclusion of light and vigorous stirring, *t*BuOH (4.0 mL, 43 mmol) and acetic acid (2.3 mL, 39 mmol) were added. The resultant mixture was stirred at 0 °C for 3 min and was then washed with Na_2CO_3 solution (20 mL) and water (20 mL) to give crude *tert*-butyl hypochlorite as a yellow oil, which was used without purification. Benzyl carbamate (9.38 g, 62.1 mmol) was dissolved in MeCN (80 mL) and added to a solution of NaOH (2.44 g, 61.0 mmol) in water (150 mL). Freshly prepared crude *tert*-butyl hypochlorite (6.9 mL, 61 mmol) was added dropwise to this solution at 15 °C over 30 min. Then, a solution of $(DHQ)_2PHAL$ (hydroquinine 1,4-phthalazinediyl diether; 780 mg, 1.00 mmol) and *tert*-butyl crotonate (3.2 mL, 20 mmol) in MeCN (70 mL) was added. The reaction was started by the addition of potassium osmate (294 mg, 0.798 mmol) and the resultant green solution was stirred at room temperature for 1.5 h. The reaction was quenched by the addition of sodium sulfite (20 g) and further stirred for 45 min. The aqueous layer was extracted with EtOAc (4×100 mL). The combined organics were washed with water (120 mL) and brine (120 mL), dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The resultant crude product was purified by flash column chromatography (petroleum ether/EtOAc, 85:15→8:2). The enantiomeric ratio (e.r.) was determined to be 94:6 by chiral HPLC (see the Supporting Information) and raised to e.r. > 99:1 by recrystallization (EtOAc/hexane) to give **21** as white needles (3.11 g, 51%). M.p.: 94 °C; $[\alpha]_D^{20} = -3.5$ ($c = 2.0$, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 1.26$ (d, $J = 7.0$ Hz, 3H, H-4), 1.44 (s, 9H, $OC(CH_3)_3$), 3.13 (d, $J = 3.8$ Hz, 1H, OH), 4.00 (dd, $J = 3.8$, 2.1 Hz, 1H, H-2), 4.26 (ddt, $J = 9.7$, 7.0, 2.1 Hz, 1H, H-3), 4.99 (d, $J = 9.7$ Hz, 1H, NH), 5.04 (d, $J = 12.3$ Hz, 1H, Cbz-H-1a), 5.08 (d, $J = 12.3$ Hz, 1H, Cbz-H-1b), 7.27–7.38 (m, 5H, aryl-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): $\delta = 18.50$ (C-4), 27.93 ($OC(CH_3)_3$), 49.11 (C-3), 66.76 (Cbz-C-1), 73.43 ($OC(CH_3)_3$), 83.72 (C-2), 128.18 (Cbz-C-3, Cbz-C-7), 128.21 (Cbz-C-5), 128.62 (Cbz-C-4, Cbz-C-6), 136.64 (Cbz-C-2), 155.62 (Cbz-C=O), 172.57 (C-1) ppm; MS (ESI): $m/z = 332.06$ $[M+Na]^+$; HRMS (ESI): calcd for $C_{16}H_{23}NO_5Na$ $[M+Na]^+$ 332.1468; found: 332.1448; IR (ATR): $\tilde{\nu} =$ 3366, 2977, 1717, 1512, 1229, 1125, 1052, 736, 696 cm^{-1}; UV (heptane): $\lambda_{max} = 207$ nm; TLC (petroleum ether/EtOAc, 4:1): $R_f = 0.20$.

tert-Butyl (2*R*,3*S*)-3-(((benzyloxy)carbonyl)amino)-2-((*tert*-butyldimethylsilyl)oxy)butanoate (22)

To a solution of **21** (1.99 g, 6.43 mmol) in pyridine (40 mL), TBDMS chloride (1.94 g, 12.9 mmol) and imidazole (569 mg, 8.36 mmol) were added and the resultant solution was stirred at room temperature for 43 h until the starting material was fully consumed (TLC monitoring). The solution was concentrated under reduced pressure, then EtOAc was added and the organic layer was washed with $NaHCO_3$ solution (3×120 mL) and brine (120 mL). The organic layer was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The resultant crude product was purified by column chromatography (petroleum ether/EtOAc, 9:1) to give **22** as a colorless oil (2.36 g, 87%). $[\alpha]_D^{20} = +13.8$ ($c = 3.7$, CH_2Cl_2); ^{1}H NMR (500 MHz, $CDCl_3$): $\delta = 0.05$ (s, 3H, $SiCH_3$), 0.10 (s, 3H, $SiCH_3$), 0.92 (s, 9H, $SiC(CH_3)_3$), 1.20 (d, $J = 6.7$ Hz, 3H, H-4), 1.42 (s, 9H, $OC(CH_3)_3$), 4.05 (d, $J = 2.3$ Hz, 1H, H-2), 4.17 (ddq, $J = 9.3$, 6.7, 2.3 Hz, 1H, H-3), 5.05 (d, $J = 12.3$ Hz, 1H, Cbz-H-1a), 5.08 (d, $J = 12.3$ Hz, 1H, Cbz-H-1b), 5.15 (d, $J = 9.3$ Hz, 1H, NH), 7.27–7.37 (m, 5H, aryl-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): $\delta = -5.46$ ($SiCH_3$), −4.65 ($SiCH_3$), 18.46 (C-4), 18.48 ($SiC(CH_3)_3$), 25.86 ($SiC(CH_3)_3$), 28.03 ($OC(CH_3)_3$), 50.27 (C-3), 66.70 (Cbz-C-1), 74.90 ($OC(CH_3)_3$), 81.81 (C-2), 128.17 (Cbz-C-5), 128.28 (Cbz-C-3, Cbz-C-7), 128.61 (Cbz-C-4, Cbz-C-6), 136.75 (Cbz-C-2), 155.71 (Cbz-C=O), 170.92 (C-1) ppm; MS (ESI): $m/z = 446.20$ $[M+Na]^+$; HRMS (ESI): calcd for $C_{22}H_{38}NO_5Si$ $[M+H]^+$ 424.2514; found: 424.2501; IR (ATR): $\tilde{\nu} = 3443$, 2930, 2857, 1723, 1500, 1214, 1130, 869, 777 cm^{-1}; UV ($CHCl_3$): $\lambda_{max} = 258$ nm; TLC (petroleum ether/EtOAc, 9:1): $R_f = 0.20$.

tert-Butyl (2*R*,3*S*)-3-(((benzyloxy)carbonyl)(methyl)amino)-2-hydroxybutanoate (23)

NaH (60% in mineral oil, 197 mg, 4.90 mmol) was suspended in THF (70 mL). Then, a solution of **22** (415 mg, 0.980 mmol) in THF (14 mL) was slowly added at room temperature. The resultant solution was stirred at room temperature for 2 h before dimethyl sulfate (1.0 mL, 10 mmol) was added and the suspension was stirred for a further 2 d. To quench the reaction, water (50 mL) was added and the resultant mixture was stirred for 40 min. Brine (75 mL) was added and the aqueous layer was extracted with EtOAc (3× 50 mL). The combined organics were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The resultant crude product was stirred in TFA and water (1:4, 25 mL) at room temperature for 3 h. The mixture was extracted with CH_2Cl_2 (3× 50 mL), the combined organics were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The resultant crude product was purified by flash column chromatography (petroleum ether/EtOAc, 85:15) to give **23** as a colorless oil (280 mg, 89% over two steps from **22**). $[\alpha]_D^{20} = -4.3$ ($c = 2.8$, CH_2Cl_2); ^{1}H NMR (500 MHz, $C_2D_2Cl_4$, 80 °C): $\delta = 1.23$ (d, $J = 6.7$ Hz, 3H, H-4), 1.39 (s, 9H, $OC(CH_3)_3$), 2.84 (s, 3H, NCH_3), 3.98 (d, $J = 6.1$ Hz, 1H, H-2), 4.17 (dq, $J = 6.7$, 6.1 Hz, 1H, H-3), 5.06 (d, $J = 12.6$ Hz, 1H, Cbz-H-1a), 5.10 (d, $J = 12.6$ Hz, 1H, Cbz-H-1b), 7.16–7.36 (m, 5H, aryl-H) ppm; ^{13}C NMR (126 MHz, $C_2D_2Cl_4$, 80 °C): $\delta = 14.60$ (C-4), 28.19 ($OC(CH_3)_3$), 31.36 (NCH_3) 54.70 (C-3), 67.39 (Cbz-C-1), 74.56 (C-2), 83.05 ($OC(CH_3)_3$), 127.71 (Cbz-C-3, Cbz-C-7), 128.14 (Cbz-C-5), 128.71 (Cbz-C-4, Cbz-C-6), 137.19 (Cbz-C-2), 156.88 (Cbz-C=O), 172.51 (C-1) ppm; MS (ESI): $m/z = 346.17$ $[M+Na]^+$; HRMS (ESI): calcd for $C_{17}H_{25}NO_5Na$ $[M+Na]^+$ 346.1625; found: 346.1612; IR (ATR): $\tilde{\nu} =$ 2977, 2938, 1691, 1451, 1399, 1319, 1248, 1141, 1025, 742, 670 cm^{-1}; UV (MeCN): $\lambda_{max} = 216$, 257 nm; TLC (petroleum ether/EtOAc, 7:3): $R_f = 0.30$.

tert-Butyl (3*S*)-2-azido-3-(((benzyloxy)carbonyl)(methyl)amino)butanoate (24)

Compound **23** (200 mg, 0.618 mmol) was dissolved in CH_2Cl_2 (13 mL). At 0 °C, triethylamine (0.19 mL, 1.4 mmol) and methanesulfonyl chloride (0.11 mL, 1.4 mmol) were added and the resultant

solution was allowed to warm to room temperature and stirred for 2 h. Subsequently, CH_2Cl_2 (40 mL) was added. The mixture was washed with brine (40 mL), dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The resultant crude mesylate (a colorless oil) was dried in vacuo for 1 h and then used without further purification. To a solution of the crude mesylate in DMF (15 mL), sodium azide (100 mg, 1.55 mmol) was added and the resultant solution was stirred at 80 °C for 21 h. The solvent was then evaporated under reduced pressure. The obtained residue was dissolved in EtOAc (30 mL) and the solution was washed with water (2×20 mL) and $NaHCO_3$ solution (20 mL). The organic layer was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The resultant crude product was purified by flash column chromatography (petroleum ether/EtOAc, 86:14) to give **24** (diastereomeric mixture) as a colorless oil (116 mg, 54% over 2 steps from **23**, d.r. 88:12 based on the ^{1}H NMR spectrum). ^{1}H NMR (500 MHz, $C_2D_2Cl_4$, 80 °C): δ = 1.23 (d, J = 6.9 Hz, 3H, H-4), 1.42 (s, 9H, $OC(CH_3)_3$), 2.85 (s, 3H, NCH_3), 3.88 (d, J = 6.8 Hz, 1H, H-2), 4.38–4.44 (m, 1H, H-3), 5.10 (s, 2H, Cbz-H-1), 7.25–7.37 (m, 5H, aryl-H) ppm; ^{13}C NMR (126 MHz, $C_2D_2Cl_4$, 80 °C): δ = 14.30 (C-4), 28.21 ($OC(\underline{C}H_3)_3$), 30.67 (NCH_3), 53.22 (C-3), 65.94 (C-2), 67.52 (Cbz-C-1), 83.50 ($O\underline{C}(CH_3)_3$), 127.93 (Cbz-C-3, Cbz-C-7), 128.32 (Cbz-C-5), 128.75 (Cbz-C-4, Cbz-C-6), 136.98 (Cbz-C-2), 155.98 (Cbz-C=O), 167.85 (C-1) ppm; MS (ESI): m/z = 371.14 $[M+Na]^+$; TLC (petroleum ether/EtOAc, 4:1): R_f = 0.35.

tert-Butyl (3*S*)-2-(((allyloxy)carbonyl)amino)-3-(((benzyloxy)-carbonyl)(methyl)amino)butanoate (25)

To a solution of the diastereomeric mixture of **24** (75 mg, 0.21 mmol) in THF and toluene (1:1, 3 mL), triphenylphosphine (165 mg, 0.629 mmol) and water (188 μL, 10.5 mmol) were added and the resultant solution was stirred at 50 °C for 22 h. Subsequently, Alloc chloride (45 μL, 0.42 mmol) and $NaHCO_3$ (35 mg, 0.42 mmol) were added and the mixture was stirred at room temperature for a further 20 h. EtOAc (40 mL) was added and the organic layer was washed with water (30 mL) and brine (30 mL), dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The resultant crude product was purified by flash column chromatography (petroleum ether/EtOAc, 4:1) to give (2*R*,3*S*)-*syn*-isomer **25a** (10 mg, 12%) as a colorless oil and the desired (2*S*,3*S*)-*anti*-isomer **25b** (76 mg, 88%) as a colorless oil. **25a**: ^{1}H NMR (500 MHz, $C_2D_2Cl_4$, 80 °C): δ = 1.18 (d, J = 6.8 Hz, 3H, H-4), 1.41 (s, 9H, $OC(CH_3)_3$), 2.77 (s, 3H, NCH_3), 4.16 (dd, J = 9.0, 9.0 Hz, 1H, H-2), 4.28–4.69 (m, 1H, H-3), 4.48 (d, J = 5.5 Hz, 2H, Alloc-H-1), 5.09 (s, 2H, Cbz-H-1), 5.15 (dd, J = 10.5, 1.3 Hz, 1H, Alloc-H-3a), 5.23 (dd, J = 17.2, 1.3 Hz, 1H, Alloc-H-3b), 5.80–5.88 (m, 1H, Alloc-H-2), 7.22–7.37 (m, 5H, aryl-H) ppm; TLC (petroleum ether/EtOAc, 4:1): R_f = 0.25. **25b**: $[\alpha]_D^{20}$ = −8.3 (c = 2.1, CH_2Cl_2); ^{1}H NMR (500 MHz, $C_2D_2Cl_4$, 80 °C): δ = 1.18 (d, J = 6.8 Hz, 3H, H-4), 1.37 (s, 9H, $OC(CH_3)_3$), 2.80 (s, 3H, NCH_3), 4.29–4.39 (m, 2H, H-2, H-3), 4.47–4.54 (m, 2H, Alloc-H-1), 5.08 (s, 2H, Cbz-H-1), 5.14–5.19 (m, 1H, Alloc-H-3a), 5.22–5.29 (m, 1H, Alloc-H-3b), 5.73–5.91 (m, 1H, Alloc-H-2), 7.18–7.38 (m, 5H, aryl-H) ppm; ^{13}C NMR (126 MHz, $C_2D_2Cl_4$, 80 °C): δ = 14.61 (C-4), 28.14 ($OC(\underline{C}H_3)_3$), 29.76 (brs, NCH_3), 53.83 (C-3), 57.88 (C-2), 66.16 (Alloc-C-1), 67.51 (Cbz-C-1), 82.89 ($O\underline{C}(CH_3)_3$), 118.02 (Alloc-C-3), 127.99 (Cbz-C-3, Cbz-C-7), 128.17 (Cbz-C-5), 128.72 (Cbz-C-4, Cbz-C-6), 133.01 (Alloc-C-2), 137.03 (Cbz-C-2), 155.88 (Alloc-C=O), 156.26 (Cbz-C=O), 170.02 (C-1) ppm; MS (ESI): m/z = 429.04 $[M+Na]^+$; HRMS (ESI): calcd for $C_{21}H_{31}N_2O_6$ $[M+H]^+$ 407.2177; found: 407.2167; IR (ATR): $\tilde{\nu}$ = 3299, 2973, 1724, 1673, 1537, 1447, 1234, 1147, 1034, 996, 760, 591 cm^{-1}; UV (MeCN): λ_{max} = 215, 257 nm; TLC (petroleum ether/EtOAc, 4:1): R_f = 0.15.

Protected L-trypotophan-L-leucine urea dipeptide TMSE ester (28)

Boc-protected tryptophan *tert*-butyl ester hydrochloride **26** (120 mg, 0.302 mmol) was dissolved in CH_2Cl_2 and aq. $NaHCO_3$ solution (1:1, 10 mL), cooled to 0 °C, and stirred vigorously. Triphosgene (40 mg, 0.13 mmol) was added in one portion and the mixture was stirred for 30 min. The aqueous layer was then extracted with CH_2Cl_2 (3×30 mL). The combined organics were dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The resultant crude isocyanate **27** was directly used in the next reaction without further purification. L-Leucine TMSE ester[21] (72 mg, 0.31 mmol) was dissolved in DMF (5 mL) and a solution of the crude isocyanate **27** in THF (6 mL) was added at room temperature. The reaction mixture was stirred at room temperature for 18 h. Subsequently, EtOAc (50 mL) was added and the mixture was washed with HCl (1 M, 3×30 mL), dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The resultant crude product was purified by flash column chromatography (petroleum ether/EtOAc, 92.5:7.5→9:1) to give **28** as a white foam (92 mg, 52% over 2 steps from **26**). $[\alpha]_D^{20}$ = +2.6 (c = 0.43, CH_2Cl_2); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 0.04 (s, 9H, $Si(CH_3)_3$), 0.90 (d, J = 6.3 Hz, 3H, Leu-H-5a), 0.92 (d, J = 6.3 Hz, 3H, Leu-H-5b), 0.96–1.07 (m, 2H, H-2′), 1.39 (s, 9H, $OC(CH_3)_3$), 1.41–1.48 (m, 1H, Leu-H-3a), 1.48–1.60 (m, 1H, Leu-H-3b), 1.59–1.72 (m, 10H, H-4, Boc-$OC(CH_3)_3$), 3.18 (d, J = 7.0 Hz, 2H, Trp-H-3), 4.16–4.26 (m, 2H, H-1′), 4.43 (ddd, J = 8.9, 8.9, 5.2 Hz, 1H, Leu-H-2), 4.67–4.78 (m, 2H, H-2′, Leu-2-NH), 4.99 (d, J = 7.7 Hz, 1H, Trp-2-NH), 7.19–7.36 (m, 2H, Trp-indole-H-5, Trp-indole-H-6), 7.39 (s, 1H, Trp-indole-H-2), 7.56–7.61 (m, 1H, Trp-indole-H-4), 8.12 (d, J = 7.2 Hz, 1H, Trp-indole-H-7) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = −1.36 ($Si(CH_3)_3$), 17.51 (C-2′), 22.06 (Leu-C-5), 23.04 (Leu-C-5), 24.85 (Leu-C-4), 28.11, 28.34 ($OC(\underline{C}H_3)_3$, Boc-$OC(\underline{C}H_3)_3$), 28.32 (Trp-C-3), 42.28 (Leu-C-3), 51.89 (Leu-C-2), 54.14 (Trp-C-2), 63.67 (C-1′), 82.34 ($O\underline{C}(CH_3)_3$), 83.60 (Boc-$O\underline{C}(CH_3)_3$), 115.23 (Trp-indole-C-7), 115.73 (Trp-indole-C-3), 119.70 (Trp-indole-C-4), 122.78 (Trp-indole-C-5), 124.27 (Trp-indole-C-2), 124.55 (Trp-indole-C-6), 131.04 (Trp-indole-C-3a), 135.48 (Trp-indole-C-7a), 149.71 (Boc-C=O), 156.60 (urea-C=O), 171.52 (Trp-C-1), 174.18 (Leu-C-1) ppm; MS (ESI): m/z = 618.41 $[M+H]^+$; HRMS (ESI): calcd for $C_{32}H_{52}N_3O_7Si$ $[M+H]^+$ 618.3569; found: 618.3565; IR (ATR): $\tilde{\nu}$ = 3368, 2959, 1729, 1551, 1370, 1252, 1153, 940, 753 cm^{-1}; UV (MeCN): λ_{max} = 229, 260, 285, 294 nm; TLC (petroleum ether/EtOAc, 9:1): R_f = 0.15.

N-Deprotected AMBA-*N*-acylated nucleosyl amino acid (30)

To a solution of protected nucleosyl amino acid **17**[12b] (170 mg, 0.291 mmol) in a mixture of DMF and THF (1:1, 5 mL), a solution of protected AMBA derivative **16** (99 mg, 0.28 mmol) in a mixture of DMF and THF (1:1, 5 mL) and 1-hydroxy-7-azabenzotriazole (HOAt; 193 mg, 1.42 mmol) were added. 1-Ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDCI; 71 mg, 0.37 mmol) was dissolved in DMF and THF (1:1, 2 mL) and *N*-methylmorpholine (NMM; 40 μL, 0.37 mmol) was added. After stirring at room temperature for 10 min, this solution was added dropwise to the aforementioned solution and the resultant reaction mixture was stirred at room temperature for 27 h. Subsequently, EtOAc (150 mL) was added and the mixture was washed with HCl (1 M, 50 mL), $NaHCO_3$ solution (3×50 mL), water (50 mL), and brine (50 mL). The organic layer was dried over Na_2SO_4 and the solvent was evaporated under reduced pressure. The resultant crude product was purified by flash column chromatography (CH_2Cl_2/MeOH, 98:2) to give the intermediate *N*-Alloc-protected AMBA-*N*-acylated nucleosyl amino acid as a white foam (245 mg, 94%). To a solution of the *N*-Alloc-protected AMBA-*N*-acylated nucleosyl amino acid (152 mg,

0.166 mmol) in CH_2Cl_2 (5 mL), phenylsilane (95 μL, 1.0 mmol) and tetrakis(triphenyl-phosphine)palladium(0) (19 mg, 17 μmol) were added under the exclusion of light. The reaction mixture was stirred at room temperature for 6 h. Subsequently, EtOAc (40 mL) was added and the mixture was washed with $NaHCO_3$ solution (40 mL), dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The resultant crude product was purified by flash column chromatography (CH_2Cl_2/MeOH, 98:2→95:5→9:1) to give **30** as a white foam (100 mg, 73%, 69% over 2 steps from **17**). $[\alpha]_D^{20} = +52.4$ ($c = 1.0$, CH_2Cl_2); ^{1}H NMR[§] (500 MHz, $CDCl_3$): $\delta = 0.04$ (s, 3H, $SiCH_3$), 0.04 (s, 3H, $SiCH_3$), 0.05 (s, 3H, $SiCH_3$), 0.06 (s, 3H, $SiCH_3$), 0.86 (s, 9H, $SiC(CH_3)_3$), 0.87 (s, 9H, $SiC(CH_3)_3$), 1.23 (d, J = 7.3 Hz, 1H, AMBA-H-4), 1.43 (s, 9H, $OC(CH_3)_3$), 1.85–2.08 (m, 2H, H-5′a, H-5′b), 2.90 (s, 3H, AMBA-NCH_3), 3.56–3.59 (m, 1H, AMBA-H-2), 3.64–3.69 (m, 1H, H-3′), 4.14–4.21 (m, 2H, H-4′, AMBA-H-3), 4.40–4.48 (m, 2H, H-2′, H-6′), 5.13–5.15 (m, 2H, Cbz-H-1), 5.39–5.49 (m, 1H, H-1′), 5.74 (d, J = 8.1 Hz, 1H, H-5), 7.25–7.38 (m, 5H, aryl-H), 7.50 (m, 1H, H-6), 7.65 (d, J = 5.8 Hz, 1H, NH) ppm; ^{13}C NMR[§] (126 MHz, $CDCl_3$): $\delta = -4.72$ ($SiCH_3$), −4.67 ($SiCH_3$), −4.48 ($SiCH_3$), −4.11 ($SiCH_3$), 13.01 (AMBA-C-4), 18.07 ($SiC(CH_3)_3$), 18.16 ($SiC(CH_3)_3$), 25.90 ($SiC(CH_3)_3$), 25.92 ($SiC(CH_3)_3$), 28.08 ($OC(CH_3)_3$), 32.05 (AMBA-NCH_3), 35.40 (C-5′), 51.89 (C-6′), 56.07 (AMBA-C-3), 58.38 (AMBA-C-2), 67.47 (Cbz-C-1), 73.78 (C-2′), 75.71 (C-3′), 82.48 ($OC(CH_3)_3$, C-4′), 93.88 (C-1′), 102.46 (C-5), 127.94, 128.08, 128.61 (Cbz-C-3–7), 136.85 (Cbz-C-2), 142.01 (C-6), 150.97 (C-2), 157.02 (Cbz-C=O), 163.35 (C-4), 170.80 (C-7′), 172.97 (AMBA-C-1) ppm; MS (ESI): m/z = 834.66 $[M+H]^+$; HRMS (ESI): calcd for $C_{40}H_{68}N_5O_{10}Si_2$ $[M+H]^+$ 834.4499; found: 834.4486; IR (ATR): $\tilde{\nu}$ = 2931, 2857, 1683, 1457, 1255, 1154, 866, 835, 776, 730 cm^{-1}; UV (MeCN): λ_{max} = 260 nm.

Protected intermediate (31)

To a solution of protected urea dipeptide **18** (40 mg, 76 μmol) in DMF (3 mL), DIPEA (32 μL, 0.18 mmol) and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP; 58 mg, 0.11 mmol) were added. The resultant solution was stirred at room temperature for 3 h. Then, a solution of *N*-deprotected AMBA-*N*-acylated nucleosyl amino acid **30** (62 mg, 74 μmol) in DMF (3.5 mL) was added and the reaction mixture was stirred at room temperature for a further 17 h. EtOAc (40 mL) was added and the mixture was washed with HCl (1 M, 30 mL) and brine (30 mL), dried over Na_2SO_4, and the solvent was evaporated under reduced pressure. The resultant crude product was purified by flash column chromatography (CH_2Cl_2/MeOH, 98:2) to give **31** as a white foam (62 mg, 64%). Owing to pronounced rotamer formation of the title compound, which resulted in very complex NMR spectra, **31** was analyzed by LC-MS only. Purity was confirmed based on the UV chromatogram of the LC-MS analysis. MS (ESI): m/z = 1334.24 $[M+H]^+$; TLC (CH_2Cl_2/MeOH, 98:2): R_f = 0.15.

Additional nucleosyl amino acid-AMBA target compound (32)

N-Deprotected AMBA-*N*-acylated nucleosyl amino acid **30** (16 mg, 19 μmol) was dissolved in TFA and water (4:1, 2.5 mL). The reaction mixture was stirred at room temperature for 25 h. Water (5 mL) was added and the solvent was evaporated by lyophilization. The resultant crude product was purified by semi-preparative HPLC (method 2) to give **32** as a white fluffy solid (9.3 mg, 74%). ^{1}H NMR (500 MHz, D_2O): $\delta = 1.41$ (d, J = 6.9 Hz, 3H, AMBA-H-4), 2.12–2.29 (m, 1H, H-5′a), 2.30–2.44 (m, 1H, H-5′b), 2.83[+], 2.87 (brs, 3H, AMBA-NCH_3), 4.02–4.21 (m, 3H, H-3′, H-4′, AMBA-H-2), 4.36 (dd, J = 5.2, 4.1 Hz, 1H, H-2′), 4.40–4.52 (m, 1H, AMBA-H-3), 4.56–4.69 (m, 2H, H-6′, AMBA-H-3[+]), 5.06–5.17 (m, 2H, Cbz-H-1), 5.75–5.83 (m, 1H, H-1′), 5.87 (d, J = 8.1 Hz, 1H, H-5), 7.36–7.50 (m, 5H, aryl-H), 7.63 (d, J = 8.1 Hz, 1H, H-6) ppm; ^{13}C NMR (126 MHz, D_2O): δ = 13.65, 14.23[+] (AMBA-C-4), 29.59[+], 30.52 (AMBA-NCH_3), 34.04 (C-5′), 50.57 (AMBA-C-3), 52.19[+], 53.14 (C-6), 55.32[+], 55.61 (AMBA-C-2), 67.98, 68.15[+] (Cbz-C-1), 72.98 (C-2′), 73.14 (C-3′), 79.82 (C-4′), 90.48 (C-1′), 102.29 (C-5), 116.41 (q, J_{CF} = 291.6 Hz, TFA-CF_3), 127.89, 128.26, 128.54, 128.83 (Cbz-C-3–7), 136.15 (Cbz-C-2), 142.05 (C-6), 151.54 (C-2), 157.84 (Cbz-C=O), 163.06 (q, J_{CF} = 35.5 Hz, TFA-C=O), 166.24 (C-4), 173.62, 173.71[+], 173.78 (C-7′, C-1′′) ppm; ^{19}F NMR (376 MHz, D_2O): $\delta = -75.56$ (TFA-CF_3) ppm; HRMS (ESI): calcd for $C_{24}H_{32}N_5O_{10}$ $[M+H]^+$ 550.2144; found: 550.2126; IR (ATR): $\tilde{\nu}$ = 1669, 1542, 1401, 1190, 1133, 808, 724, 557 cm^{-1}; UV (HPLC, MeCN/H_2O): λ_{max} = 216, 261 nm; HPLC (method 2): t_R = 16.0 min.

Additional urea dipeptide target compound (33)

Protected urea dipeptide **18** (11 mg, 22 μmol) was dissolved in TFA and water (4:1, 2.5 mL). The reaction mixture was stirred at room temperature for 26 h. Water (5 mL) was added and the solvent was evaporated by lyophilization. The resultant crude product was purified by semi-preparative HPLC (method 1) to give **33** as a yellowish fluffy solid (4.1 mg, 51%). ^{1}H NMR (500 MHz, CD_3OD): $\delta = 0.93$ (d, J = 6.4 Hz, 3H, Leu-H-5a), 0.95 (d, J = 6.6 Hz, 3H, Leu-H-5b), 1.50 (ddd, J = 13.7, 9.8, 5.5 Hz, 1H, Leu-H-3a), 1.58 (ddd, J = 13.7, 8.7, 5.0 Hz, 1H, Leu-H-3b), 1.67–1.78 (m, 1H, Leu-H-4), 3.23 (ddd, J = 14.7, 5.9, 0.6 Hz, 1H, Trp-H-3a), 3.23 (ddd, J = 14.7, 5.6, 0.6 Hz, 1H, Trp-H-3b), 4.28 (dd, J = 9.8, 5.0 Hz, 1H, Leu-H-2), 4.61 (dd, J = 5.8, 5.6 Hz, 1H, Trp-H-2), 7.00 (ddd, J = 8.0, 7.0, 1.0 Hz, 1H, Trp-indole-H-6), 7.00 (ddd, J = 8.1, 7.0, 1.1 Hz, 1H, Trp-indole-H-5), 7.12 (s, 1H, Trp-indole-H-2), 7.31 (ddd, J = 8.1, 8.0, 1.0 Hz, 1H, Trp-indole-H-4), 7.58–7.60 (m, 1H, Trp-indole-H-7) ppm; ^{13}C NMR (126 MHz, CD_3OD): δ = 22.01 (Leu-C-5a), 23.39 (Leu-C-5b), 25.93 (Leu-C-4), 29.07 (Trp-C-3), 42.49 (Leu-C-3), 52.65 (Leu-C-2), 54.95 (Trp-C-2), 110.57 (Trp-indole-C-3), 112.07 (Trp-indole-C-4), 119.63 (Trp-indole-C-7), 119.80 (Trp-indole-C-6), 122.28 (Trp-indole-C-5), 124.71 (Trp-indole-C-2), 129.09 (Trp-indole-C-3a), 137.99 (Trp-indole-C-7a), 159.98 (urea-C=O), 176.10 (Trp-C-1), 177.27 (Leu-C-1) ppm; MS (ESI): m/z = 362.15 $[M+H]^+$; HRMS (ESI): calcd for $C_{18}H_{24}N_3O_5$ $[M+H]^+$ 362.1710; found: 362.1703; IR (ATR): $\tilde{\nu}$ = 3380, 2958, 1640, 1556, 1449, 1192, 746 cm^{-1}; UV (HPLC, MeCN/H_2O): λ_{max} = 221, 280 nm; HPLC (method 1): t_R = 16.0 min.

Additional full-length target compound (34)

Protected intermediate **31** (20 mg, 15 μmol) was dissolved in TFA and water (4:1, 2.5 mL). The reaction mixture was stirred at room temperature for 42 h. Water (6 mL) was added and the solvent was evaporated by lyophilization. The resultant crude product was purified by semi-preparative HPLC (method 3) to give **34** as a white fluffy solid (4.3 mg, 31%). ^{1}H NMR (500 MHz, CD_3OD): $\delta = 0.91$ (d, J = 6.6 Hz, 3H, Leu-H-5a), 0.95 (d, J = 6.9 Hz, 3H, Leu-H-5b), 1.13[+], 1.18 (d, J = 6.8 Hz, 3H, AMBA-H-4), 1.42–1.49 (m, 1H, Leu-H-3a), 1.50–1.57 (m, 1H, Leu-H-3b), 1.61–1.72 (m, 1H, Leu-H-4), 2.06–2.16 (m, 1H, H-5′a), 2.23–2.31 (m, 1H, H-5′b), 2.83 (s, 3H, NCH_3), 3.19 (dd, J = 14.8, 6.7 Hz, 1H, Trp-H-3a), 3.27 (dd, J = 14.8, 5.1 Hz, 1H, Trp-H-3b), 3.88 (dd, J = 5.5, 5.1 Hz, 1H, H-3′), 4.00–4.08 (m, 1H, H-4′), 4.10 (dd, J = 4.8, 4.8 Hz, 1H, H-2′), 4.21 (dd, J = 9.8, 4.8 Hz, 1H, Leu-H-2), 4.34–4.43, 4.46–4.55[+] (m, 1H, AMBA-H-3), 4.56–4.65 (m, 2H, H-6′, Trp-H-2), 4.66–4.72 (m, 1H, AMBA-H-2), 5.06 (d, J = 12.4 Hz, 1H, Cbz-H-1a), 5.10 (d, J = 12.4 Hz, 1H, Cbz-H-1b), 5.68 (d, J = 8.1 Hz, 1H, H-5), 5.78 (d, J = 4.8 Hz, 1H, H-1′), 7.00 (ddd, J = 8.0, 7.0, 1.0 Hz, 1H, Trp-indole-H-6), 7.03–7.08 (m, 1H, Trp-indole-H-5), 7.11[+], 7.13 (s, 1H, Trp-indole-H-2), 7.23–7.42 (m, 6H, Cbz-aryl-H, Trp-indole-H-4), 7.56 (d, J = 8.0 Hz, 1H, Trp-indole-H-7), 7.60 (d, J =

8.1 Hz, 1H, H-6), 8.08–8.17 (m, 1H, 6′-NH or Trp-NH), 8.21–8.30 (m, 1H, AMBA-NH) ppm; ^{13}C NMR (126 MHz, CD_3OD): δ = 14.42, 14.96^{+} (AMBA-C-4), 22.02 (Leu-C-5), 23.53 (Leu-C-5), 25.85 (Leu-C-4), 29.02 (Trp-C-3), 30.75 (NCH_3), 36.02 (C-5′), 42.17 (Leu-C-3), 50.96 (Trp-C-2), 53.87 (Leu-C-2), 54.86 (C-6′), 54.86^{+}, 54.93 (AMBA-C-3), 56.92 (AMBA-C-2), 68.37 (Cbz-C-1), 74.59 (C-2′), 74.88 (C-3′), 81.74 (C-4′), 91.74 (C-1′), 102.95 (C-5), 110.63 (Trp-indole-C-3), 112.20 (Trp-indole-C-4), 119.50 (Trp-indole-C-7), 119.83 (Trp-indole-C-6), 122.34 (Trp-indole-C-5), 124.77 (Trp-indole-C-2), 128.66, 128.91, 128.98 (Cbz-C-3–7), 129.49 (Trp-indole-C-3a), 137.99 (Trp-indole-C-7a), 138.18 (Cbz-C-2), 142.76 (C-6), 152.25 (C-2), 158.15 (Cbz-C=O), 159.89 (urea-C=O), 166.14 (C-4), 171.49 (AMBA-C-1), 174.10 (C-7′), 175.91 (Trp-C-1), 176.22 (Leu-C-1) ppm; HRMS (ESI): calcd for $C_{42}H_{53}N_8O_{14}$ $[M+H]^+$ 893.3676; found: 893.3659; IR (ATR): $\tilde{\nu}$ = 2955, 1663, 1549, 1456, 1192, 1129, 745 cm^{-1}; UV (HPLC, MeCN/H_2O): λ_{max} = 220, 260 nm; HPLC (method 3): t_R = 28.5 min.

MraY assay

The overexpression of MraY and the fluorescence-based assay for MraY activity were performed as described before.[23d,e] In brief, a crude membrane preparation of MraY from *S. aureus* (1 μL, final overall protein concentration ~1 mg mL^{-1})[23d,e] was added to a mixture of undecaprenyl phosphate (50 μM), dansylated Park's nucleotide (7.5 μM),[23d] and the tested compound in buffer (100 mM Tris-HCl buffer pH 7.5, 200 mM KCl, 10 mM $MgCl_2$, 0.1% Triton X-100, 20 μL overall). Fluorescence of the assay mixtures was measured over time (plate reader, 384-well plate format, λ_{ex} = 355 nm, λ_{em} = 520 nm). MraY activity at a specific inhibitor concentration was calculated by linear regression (0 to 2 min) and then plotted against logarithmic inhibitor concentrations (with sigmoidal fit to obtain IC_{50} values).

Antibacterial testing

Tests for antibacterial activity were performed as described before.[10]

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft (DFG, grant DU 1095/5-1) and the Fonds der Chemischen Industrie (FCI, doctoral fellowship to G.N.) for financial support and Jannine Ludwig and Martina Jankowski (Saarland University) for technical assistance. Open access funding enabled and organized by Projekt DEAL.

Keywords: antibiotics · hybrid structures · natural products · nucleosides · structure–activity relationship

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Manuscript received: July 18, 2020
Revised manuscript received: September 7, 2020
Accepted manuscript online: September 8, 2020
Version of record online: November 16, 2020